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(hERR 1) in Breast Cancer and Hormonally Insensitive
Disease

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13. ABSTRACT (Maximum 200 Words) A plausible candidate for a novel breast cancer prognosticator and target for therapy is the estrogen-related receptor α (ERR α), an orphan nuclear receptor that shares significant sequence identity with estrogen receptors (ERs) but does not bind estrogens. The goals of these studies are to assess ERR α 's utility as a novel breast cancer prognosticator and to elucidate ERR α 's role in modulating estrogen-responsive gene transcription. The data obtained to date indicate that: (i) ERR α is itself an estrogen-responsive gene (ii) ERR α expression levels are significantly lower in a small panel of breast carcinomas relative to normal mammary tissues, (iii) the ERR α /ER α ratio significantly correlates with S-phase fraction and DNA ploidy within the tumors, (iv) ERR α represses estrogen-stimulated transcription in MCF-7 cells, (v) ERR α may modulate expression of numerous estrogen-responsive genes whose promoters contain identified ERR α -binding sites, and (vi) ERR α -dependent transcription can be activated through increased c-erbB2 signaling, a gene implicated in tamoxifen resistance. These findings may provide the basis for future studies to determine whether ERR α status may indicate sensitivity to hormonal therapies, progression to tamoxifen resistance, and disease outcome.				
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INTRODUCTION

Approximately 50 % of breast cancer patients with estrogen receptor α (ER α) -positive tumors respond to antiestrogen therapy (1). Since expression of progesterone receptor (PgR) is dependent on ER α activity, further selection of patients for ER α -positive and PgR-positive tumors enhances the breast cancer hormonal therapy response rate to nearly 80 % (2). Estrogen-related receptor α (ERR α), an orphan nuclear receptor that shares significant sequence identity with ER α and ER β but does not bind estrogens (3), has been shown by my research group (4, 5) and others (6-9) to bind and activate transcription through estrogen response elements (EREs) and extended steroid receptor half-sites previously identified as steroidogenic factor 1 (SF-1) response elements (SFREs). Recently, ER α has been shown to activate transcription through SFREs as well as EREs (9). My research group [Fig. 5; O'Reilly, Ariazi, and Mertz, in preparation; (4)] and Yang *et al.* (10) have also demonstrated that ERR α can interact via direct protein-protein contacts with ER α . Hence, there are several mechanisms whereby ERR α may modulate estrogen responsiveness through modulating or substituting for ER α activities, making it a plausible candidate for a novel breast cancer prognosticator and target for therapy. The goals of this Post-doctoral Fellowship are: (i) to ascertain alterations which regulate ERR α activities and if these alterations impact the etiology of some breast cancers; (ii) to determine if ERR α impacts the phenotype of hormone-responsive and non-responsive breast tumor cells; and (iii) to elucidate the possible mechanism(s) by which ERR α modulates the transcriptional regulation of estrogen-responsive genes implicated in breast cancer.

BODY

Specific Aim I - To test whether alterations in expression, RNA splicing, phosphorylation status, subcellular localization, or mutations in ERR α significantly correlate with the development of some breast cancers. (A) Assays will be developed with human mammary carcinoma MCF-7 cell derivatives in Aim II to characterize ERR α RNA and protein abundance, possible splicing variants, possible phosphorylation isoforms, subcellular localization, and possible mutations. (B) These assays will be used to characterize clinical ER α -positive primary breast carcinomas for ERR α . (C) Additionally, ER α -positive/tamoxifen-resistant breast tumors will be examined for ERR α RNA expression, splicing variants, and possible mutations.

Task 1. To test whether alterations in ERR α significantly correlate with the development of clinical ER α -positive primary breast carcinomas and ER α -positive/tamoxifen-resistant breast tumors (months 1-36)

Patients with breast tumors that express ER α have an improved prognosis relative to patients with ER α -negative breast tumors (1, 2, 11). Given that ER α and ERR α share multiple biochemical activities including binding to many of the same transcriptional response elements to modulate estrogen responsiveness, I hypothesize that typing breast tumors for ERR α levels could further improve ER α 's prognostic value.

Task 1A. Develop assays with breast cancer cell lines to look for alterations in ERR α (months 1-12).

Development of real-time quantitative polymerase chain reaction (PCR) assays.

To begin to test whether ERRs are involved in breast carcinogenesis through modulation of estrogen responsiveness, I initially determined whether ERRs themselves are estrogen-responsive genes. I also investigated whether breast carcinomas exhibit differences in expression of ERRs relative to normal mammary epithelial cells and whether expression levels of these genes among the tumors correlate with ER and PgR status and clinical properties indicative of tumor aggressiveness. To measure expression of ERR α , ERR β , ERR γ , ER α , ER β , and PgR, real-time quantitative PCR assays were developed. In these assays, continuous measurement of fluorescence due to specific complex formation of Sybr Green I with double-stranded PCR products versus single-stranded DNA facilitates accurate and sensitive quantitation of initial mRNA molar amounts (12-14). My assays were developed using an ABI 7700 sequence detection system (Applied Biosystems). Because PCR efficiency decreases with increasing number of cycles due to limiting polymerase, nucleotides, and primers, the critical parameter recorded in the real-time PCR assay is the threshold cycle (C_t), *i.e.*, the cycle (measured to a fractional value of the 1 minute extension phase of the PCR) when PCR products are initially detected over background fluorescence. Background fluorescence levels are determined from the signal intensity of no-template control reactions. Spectral compensation of signal intensity differences among sample wells is accomplished by the inclusion of ROX, a fluorescent reporter dye, in the enzyme reaction buffer (Perkin Elmer). In my real-time PCR assays, each transcript's initial copy number in an unknown sample was calculated based on regression analysis of its corresponding C_t standard curve, which was generated by serial dilutions covering 6 logs of known starting amounts of the target template in every experiment. PCR primers were optimized for high efficiency, *i.e.*, product size smaller than 125 bp and no spurious bands. All RNA samples were pre-treated with DNase to eliminate contaminating cellular DNA. To minimize non-specific product amplification, the real-time PCR assays employed AmpliTaq Gold (Perkin Elmer), a chemically modified form of Taq polymerase that is completely inactive at room temperature and requires an initial 10-minute heat activation step at 95° C; thus, the use of this enzyme results in an automatic "hot start". Specific PCR products were verified by analysis of DNA melting curves (15). To control for variability in RNA integrity and cDNA synthesis efficiency, assays were normalized to the estradiol (E_2)-independent 36B4 gene, previously identified as the acidic ribosomal phosphoprotein P0 gene (16).

Effects of estrogens and antiestrogens on expression of ERRs.

Because the effects of estrogens and antiestrogens on ER α and PgR mRNA expression in MCF-7 cells are well documented, I used this model system to develop and verify my real-time quantitative PCR assays (Fig. 1). I found, as expected, that E_2 repressed ER α expression while it induced PgR

expression (17-20). Also expected, the partial antiestrogen 4-hydroxy tamoxifen (4OHT) did not significantly affect ER α expression (20), though it increased PgR expression to a lesser degree than E₂, likely because 4OHT does not block ER α 's AF-1 transactivation domain (21, 22). The complete antiestrogen ICI-182780 treatment increased ER α mRNA expression and did not change PgR expression. Taken together, these data demonstrate that the real-time quantitative PCR assays are working properly.

Interestingly, I found that E₂ significantly induced, while 4OHT probably repressed ERR α mRNA expression. Induction of ERR α mRNA levels by the synthetic estrogen diethylstilbestrol (DES) has been independently confirmed in murine endometrium *in vivo* (23). Thus, ERR α is responsive to estrogens. Given that ERs and ERRs bind both EREs and SFREs [see Figs. 3, 9 and 11 below; (4, 8, 9)], my finding that the ERR α /ER α ratio changed 45-fold with E₂ treatment relative to the control conditions is especially important. This dramatic change in the ERR α /ER α ratio could lead to significant changes in response element occupancy which could, in turn, modulate estrogen responsiveness. In support of this hypothesis is my finding in transient transfection assays that an increase in the ERR α /ER α ratio in MCF-7:WS8 cells leads to antagonism of E₂-stimulated transcription (see Fig. 6B below). Thus, the increased ERR α /ER α molar ratio that occurs in E₂-treated cells may lead to antagonism of endogenous estrogen-responsive gene transcription, thereby acting as a negative feedback on regulation of E₂ signaling. I also found that the ERR α and ER α mRNAs were present at comparable levels in MCF-7:WS8 cells, while ER β and ERR β mRNA levels were very low (data not shown). The finding of very low ER β levels is also consistent with an independent report (24), further validating the real-time PCR assays.

Screening for ERR α mRNA splice-variants and mutations in cell lines.

Many variant ER α mRNAs arising from alternative splicing (25-28) and mutations (29-33) have been identified in breast carcinomas and postulated to be involved in the development of the disease. To determine whether some breast carcinomas contain ERR α 1 mRNA splice-variants as evidenced by changes in the size of ERR α cDNA fragments, a series of twelve overlapping reverse transcriptase (RT)-PCR primers were designed spanning ERR α 1's 5' untranslated region (UTR) and its complete open reading frame (ORF). These primer sets span exon-intron junctions to prevent the amplification of potential contaminating genomic DNA. The RT-PCR primers were used in various combinations to amplify ERR α 1's 5' UTR and ORF in six overlapping ~350-bp cDNA fragments and to amplify its entire ORF in a single 1325-bp fragment. Initially, I tested my RT-PCR assays with established mammary cell lines. I found that ERR α 1 mRNA is expressed and its structure is normal in ER α -positive MCF-7:WS8, MCF-7:5C and T47D cells, and ER α -negative MDA-MB-468 and MDA-MB-231 cells (data not shown). However, ERR α 1 mRNA abundance appeared lower in MDA-MB-231 cells compared to the other cell lines. I also examined these cell lines for ERR α 2 mRNA using a primer that anneals to its unique 5' region. An ERR α 2-specific cDNA fragment of correct size was amplified in 3 of the 4 mammary cell lines; interestingly, the MDA-MB-231 cells were devoid of ERR α 2 mRNA (data not shown). As a control for cDNA integrity, I confirmed the presence of 36B4 mRNA in all of the cell lines. Thus, ERR α mRNA abundance is low in at least one ER α -negative mammary carcinoma cell line.

To identify mutations in ERR α and to discern the fine structure of alternatively spliced ERR α mRNA species, I have developed automated sequencing protocols using these same series of diagnostic ERR α mRNA RT-PCR primer sets. Automated DNA sequencing equipment is readily available as a core facility at the McArdle Laboratory. Initially, the sequencing protocols were developed using cDNAs derived from normal mammary gland and uterine tissues. I found a 3-bp insertion (CAG) between nucleotides 745 and 746 that introduces an additional Gln amino acid relative to the ERR α sequence deposited in Genbank by C.T. Teng's research group [Accession No. L38487; (10)]. The insertion of 3 bases in ERR α has been independently confirmed (34). Additionally, I have sequenced and confirmed the 3-base CAG insertion in ERR α from the same endometrial RL95-2 cells that C.T. Teng's group used to determine ERR α 's sequence. Furthermore, I sequenced ERR α from MCF-7:WS8, MCF-7:5C, T47D, MDA-MB-231, MDA-MB-468, and HeLa cells, always finding the same 3-base insertion. Accordingly, it is likely the 3-base CAG insertion exists in the wild-type ERR α sequence.

Development of ligand affinity-purified polyclonal anti-ERR α antibodies.

I needed ERR α -specific antisera to be able to examine breast tissues for ERR α protein expression by immunohistochemistry (IHC) as proposed in above and to identify definitively and to characterize ERR α -binding sites as proposed in **Revised Aim IIIA**. Because the lab group's previous α -GST-ERR α 1₁₇₋₃₂₉ antibody interfered with ERR α 's ability to bind DNA in EMSAs [see Fig. 3; (4)], I raised new anti-ERR α antibodies. ERR α amino acid sequences were evaluated for predicted antigenicity, hydropathy, and surface exposure using the Protean program of the DNASTAR bioinformatics package (Madison, WI). Basic local alignment search tool (BLAST) analyses (35) allowed further selection of candidate immunogenic peptides; queries showing an exact sequence match with only ERR α and not other family members were chosen. The amino acid sequences were each synthesized in the form of multiple antigen peptides (MAPs) or eight peptide copies attached to a non-immunogenic polylysine core and used to immunize New Zealand white male rabbits. Polyclonal antibodies were ligand-affinity purified from the collected rabbit serum using sepharose columns coupled to each immunizing-ERR α MAP and given designations corresponding to the respective location of the immunizing peptide: anti-ERR α 1₁₈₋₃₂, anti-ERR α 1₄₉₋₆₆, and anti-ERR α 2₃₁₋₄₅, the last of which is specific to the unique N-terminal region in ERR α 2. The anti-ERR α 1₄₉₋₆₆ antibodies were found to be suitable for Western blotting applications (Fig. 2) and immunoshifting ERR α -DNA complexes in EMSAs (Fig. 3).

Studies on ERR α phosphorylation and development of monoclonal anti-ERR α phosphospecific antibodies are discussed in Task 2C.

Task 1B. Examine primary ER α -positive breast carcinomas (months 2-24).

Expression of ERRs in breast tissues and correlations with clinicopathological factors.

ER α levels are usually low in normal mammary epithelium and high in breast tumor cells (36-41). This alteration in ER α expression is likely an important etiologic event in breast carcinogenesis [reviewed in (42)]. Thus, I examined clinical breast tumors and normal mammary epithelial cells (MECs) for expression of nuclear receptor mRNAs to confirm that my assays detect this change in ER α expression and to test whether expression of ERRs is also altered between these tissues. The breast carcinomas were obtained from the National Breast Tissue Resource (SPORE) at The Baylor College of Medicine through Dr. Gary Clark in a blinded fashion regarding clinical breast tumor properties, including ER and PgR status as measured by biochemical ligand-binding assays (ER-LB and PgR-LB, respectively), and cellular S-phase fraction and DNA ploidy as determined by flow cytometry analysis. For my preliminary experiments, I used as a control normal MECs derived from mammoplasty reduction surgeries obtained from the University of Wisconsin-Madison through Dr. Colin Jefcoate. Because it is technically very difficult to isolate high quality RNA from such a fatty tissue, bulk mammary gland tissue was enzymatically dispersed with collagenase and subjected to differential centrifugation to enrich and collect MECs. Dr. Jefcoate's lab supplied us with MECs after expansion in culture for a single passage under defined culture conditions that supported the outgrowth of both luminal and basal MECs (43, 44).

Using the real-time quantitative PCR assays, I have determined to date expression levels of ER α , PgR, ER β , ERR α , and ERR β in a small panel of 8 clinical random primary breast carcinomas and normal MECs derived from 3 individual mammoplasty reductions (Fig. 4). I evaluated whether a given gene's mRNA expression levels were significantly different in normal MECs as a group compared to the breast carcinomas as a group using the Wilcoxon non-parametric two-sided rank sum test (45). I found ER α levels were significantly higher ($p=0.01$), while ER β levels were significantly lower ($p=0.05$) in breast carcinomas compared to normal MECs. My findings are consistent with previous reports that ER α mRNA levels are significantly higher (36) and ER β levels are significantly lower in breast carcinomas than normal mammary glands (37, 46, 47), though ER β is sometimes co-expressed with ER α in breast tumors (48) albeit at much lower levels than ER α (49). Thus, my real-time PCR assays again show expected results.

I also found that ERR α ($p=0.02$) and ERR β ($p=0.01$) were significantly lower in breast carcinomas relative to normal MECs by the Wilcoxon rank sum test. These significant differences in expression of ERR α and ERR β suggest that these receptors may also be involved in the development of breast cancer. Of potential importance, the ERR α /ER α ratio was quite significantly lower in breast

carcinomas relative to MECs [22-fold ($p=0.01$) comparing means; 48-fold comparing medians]. This large difference in the relative ratio of these two receptors probably has important implications in estrogen responsiveness since, as discussed above, it is likely that the $ERR\alpha/ER\alpha$ ratio largely determines which receptor preferentially occupies EREs and SFREs. Thus, many sites usually occupied by $ERR\alpha$ in normal mammary epithelium may be primarily occupied by $ER\alpha$ in breast carcinomas, thereby resulting in differential expression of these estrogen-responsive genes. I will test further whether $ERR\alpha$ and the $ERR\alpha/ER\alpha$ ratio are significantly lower in breast carcinomas than MECs. Even though the MECs used here retain expression of differentiation markers (43), it is possible that their gene expression profiles do not fully reflect the gene expression profiles of MECs *in vivo* due to their having been expanded through a single cell-culture passage. Hence, I will use in my future studies normal MECs enzymatically dispersed from bulk mammary gland tissues, but not expanded in culture.

Next, I assessed for the carcinomas whether correlations existed between mRNA levels of each nuclear receptor and any previously determined clinicopathological factors. I were unblinded to the breast tumors' clinical biomarkers following the completion of my assays. The non-parametric Spearman's Rank Correlation Coefficient, designated ρ_s (Spearman's rho) (45), was used as a measure of relationships between breast carcinoma properties and mRNA levels of each gene. Absolute values of ρ_s near 1 or 0 indicate a strong or weak correlation, respectively, while the sign indicates a positive or negative relationship. Because the DNA ploidy data were in the form of dichotomous observations, tumors containing aneuploid or diploid nuclei were assigned ranks of 1 and 2, respectively. Raw data were used for ranking all other parameters and mRNA levels. Spearman's rhos for pairwise correlations of breast carcinoma properties are summarized in Table 1.

While the findings presented here are quite preliminary due to the small sample size, the data indicate some intriguing trends. $ER\alpha$ mRNA abundance as determined by real-time quantitative PCR showed a strong and significant correlation ($\rho_s = 0.83$, $P = 0.01$) with ER protein levels as determined by the ligand-binding assay (ER-LB), which classified 7 of the 8 tumors as ER-positive. Similarly, Chevillard and colleagues (50) found that a greater number of breast tumors were $ER\alpha$ -positive by semi-quantitative RT-PCR (86 of 105 samples) than by standardized enzymeimmunoassays (EIA; 79 of 105 samples). These discrepancies may be due to alterations in $ER\alpha$ that abrogate its ability to bind ligand; such mutations in $ER\alpha$'s LBD have been documented to occur in breast tumors [reviewed in (33)]. These discrepancies may also be explained by the heterogeneous nature of tumor cell subpopulations and tissue sampling error. Nonetheless, the strong correlation between $ER\alpha$ mRNA levels and ER-LB provides further support that the real-time quantitative PCR assays are working as expected.

$ERR\alpha$ mRNA levels did not correlate with expression of other genes or clinical properties. The $ER\alpha$ mRNA and ER-LB levels also did not correlate with other clinical biomarkers. Nevertheless, the $ERR\alpha/ER\alpha$ ratio correlated with ER-LB ($\rho_s = -0.76$, $P < 0.05$), $ER\alpha$ expression ($\rho_s = 0.88$, $P < 0.05$), and PgR expression ($\rho_s = -0.81$, $P < 0.05$). The potential clinical importance of the $ERR\alpha/ER\alpha$ ratio is illustrated by its significant correlations with DNA ploidy ($\rho_s = -0.85$, $P < 0.05$) and S-phase fraction ($\rho_s = 0.71$, $P < 0.10$). These preliminary findings provide actual support to the hypothesis of the prognostic importance of the $ERR\alpha/ER\alpha$ ratio.

$ERR\beta$ mRNA levels correlated with $ER\beta$ mRNA levels ($\rho_s = 0.81$, $P < 0.05$), suggesting a possible functional relationship between these genes as well. It has been reported that $ER\beta$ -positive tumors are associated with clinical indicators of breast cancer (47, 48). Likewise, it is possible that $ERR\beta$ levels may also have clinical significance. I plan to follow up on this finding by continuing to analyze tumor samples for $ERR\beta$ mRNA levels along with $ERR\alpha$ in the experiments proposed in **Aim IB**. However, biochemical and functional analysis of $ERR\beta$ is beyond the scope of this application and is the focus of a separate, small "concept" grant supported by the Department of Defense (BC995935).

Screening for $ERR\alpha$ mRNA splice-variants and mutations in breast tumors.

The diagnostic RT-PCR assays for characterization of $ERR\alpha$'s mRNA structure as described in **Task 1A** were used to examine 9 random clinical breast carcinomas. The RT-PCR assays indicated that $ERR\alpha 1$ was expressed and its mRNA was properly spliced in all of the tumors (data not shown). The $ERR\alpha 2$ -specific primers also enabled amplification of a PCR product of correct size in all 9 tumors, though a second round of PCR amplification was required to detect $ERR\alpha 2$ in 2 of the 9 tumors [Fig. 5

(round 1 of PCR); data not shown (round 2 of PCR)]. Interestingly, in one breast carcinoma (number 9), an additional variant ~90 bp larger than the expected $ERR\alpha 2$ product was detected. DNA sequence analysis of the larger PCR product indicated that it was an mRNA splice-variant with a precise insertion of part of $ERR\alpha 1$'s intron 1 sequence at the exon-1/2 splice junction. Therefore, at least one $ERR\alpha$ splice variant likely exists in breast tumor cells.

Task 1C. Examine wild-type $ER\alpha$ /tamoxifen-resistant breast tumors (months 25-36).

No progress on the examination of wild-type $ER\alpha$ /tamoxifen-resistant breast tumors has as yet been accomplished.

Specific Aim II - To test whether the genetic ablation of $ERR\alpha$ affects cellular growth and death characteristics following estrogen and antiestrogen treatment of matched MCF-7 cell clones which are either sensitive (i.e., MCF-7:WS8 cells) or insensitive (i.e., MCF-7:5C cells) to these drugs. (A) Because the ligand for $ERR\alpha$ is not yet identified, somatic cell knockouts of the $ERR\alpha$ gene in MCF-7:WS8 and MCF-7:5C cells will be generated. (B) The resulting $ERR\alpha$ knockout cells and their parental $ERR\alpha$ wild-type cells will be characterized for cellular growth characteristics and apoptosis after treatment with estrogen and antiestrogens, as well as examined in standard cell transformation assays.

Task 2. To knockout the $ERR\alpha$ gene in the matched hormone responsive and non-responsive cell lines, MCF-7:WS8 and MCF-7:5C cells, respectively, and characterize possible alterations in cellular growth and apoptosis after treatment of these cells with estrogens and antiestrogens (months 1-24)

Task 2A. Determination of $ERR\alpha$ copy number by karyotype coupled with FISH analysis (months 1-6).

Task 2B. Generation of $ERR\alpha$ somatic cell knockouts using MCF-7:WS8 and MCF-7:5C cells (months 7-18).

Task 2C. Characterization of $ERR\alpha$ genetic ablation on cellular growth and apoptosis of MCF-7:WS8 and MCF-7:5C cells following treatment with estrogen and antiestrogens (months 19-24).

In my original fellowship application, I hypothesized that $ERR\alpha$ may play a role in the development of TAM-resistant breast cancer. As a model system of TAM resistance, I proposed to characterize the matched hormonally sensitive MCF-7:WS8 and insensitive MCF-7:5C cells for potential alterations in $ERR\alpha$ -dependent transcription. These cell sublines were generated by long-term growth selection of MCF-7 parental cells cultured under estrogenized and estrogen-free conditions, respectively (51). Pilot transient-transfection and ERE-driven luciferase reporter gene assays were developed to initially address $ERR\alpha$'s transactivation potential in the MCF-7:WS8 and MCF-7:5C cells. These studies showed that $ERR\alpha$ interfered with basal and E_2 -stimulated transcription in the WS8 cells (Fig. 6B, discussed in detail in **Revised Task 3B**). In contrast, the hormonally insensitive 5C cells did not exhibit significant E_2 -stimulation of ERE-mediated reporter gene activity as expected. If alterations in $ERR\alpha$'s transcriptional potential were involved in the TAM-resistant phenotype in 5C cells, one may predict that $ERR\alpha$ would activate ERE-mediated transcription in these cells rather than repress transcription as in the WS8 cells. However, the presence of an $ERR\alpha$ expression plasmid did not significantly affect the very low basal reporter activity in these 5C cells (data not shown). Moreover, as described above in **Task 1A**, 5C cells do not express $ERR\alpha$ splice-variant mRNAs or exhibit mutations in $ERR\alpha$ cDNA coding sequence. These preliminary data suggest that factors other than $ERR\alpha$ are involved in the development of TAM-resistance in this particular model system. However, given that $ERR\alpha$ likely modulates estrogen-responsive transcription, $ERR\alpha$ may still play a role in the development of a significant number of TAM-resistant breast cancers, though not in all cases. Hence, a better-defined model system of TAM-resistant breast cancer other than matched WS8 and 5C cells may be better suited for exploring $ERR\alpha$'s potential role in TAM-resistant disease progression.

Overexpression of epidermal growth factor receptor (EGFR) (52) and *c-erbB2* [also termed HER-2 and *neu*; (53, 54)] has been implicated in the failure of antiestrogen therapy in some breast

cancer patients. Several reports describe the reproducibility of TAM-stimulated growth of MCF-7 cells as solid tumors in athymic nude mice (55-58). These tumors overexpressed EGFR relative to normal MCF-7 cells (59). Elevated MAPK activity was demonstrated in T5-PRF cells, another mammary carcinoma cell line growth-selected for estrogen independence from parental estrogen-responsive T5 human breast cancer cells (60). Stable introduction of *c-erbB2* into MCF-7 cells conferred TAM resistance (61, 62). Both EGFR and *c-erbB2* signal through the Ras/MAPK pathway and MAPK signaling mediates activation of unliganded ER α through phosphorylation events (62-66). Because *c-erbB2* can induce activation of unliganded ER α , I tested whether increased *c-erbB2* signaling could also modulate ERR α 's transcriptional potential using transient-transfection and reporter gene assays (Fig. 7). These assays employed a matched set of plasmids that encode rat versions of oncogenic *c-erbB2* (*c-erbB2*_{Onc}, pJRneu) and dominant-negative *Hras* (*Hras*_{DN}, pJRHras_{DN}) under the control of a retroviral promoter [(67-69); obtained from Michael N. Gould's laboratory]. MCF-7:WS8 cells were co-transfected with the indicated combinations of plasmids and incubated in the presence or absence of the antiestrogen ICI-182780 to distinguish between effects of endogenous ER α and ERR α . I found that an increased level of ERR α 1 by itself had no significant effect on SFRE-driven transcription (Fig. 7, 2 vs. 1), but did activate transcription in the presence of *c-erbB2*_{Onc} (Fig. 7, 4 vs. 2), with this activation being attenuated when *Hras*_{DN} was present as well (Fig. 7, 7 vs. 4). These data indicate that increased MAPK signaling may activate ERR α -dependent transcription. Next, I constructed an ERR α 1 truncation mutant (ERR α 1₁₋₃₇₆) that lacks a conserved coactivator binding site termed a nuclear receptor (NR) box in ERR α 's C-terminal putative AF2 domain (70). NR boxes, comprised of the consensus sequence LXXLL [where L is leucine and X is any amino acid], mediate protein-protein interactions between NRs and coactivators, leading to recruitment of histone acetylation complexes and the basal transcription machinery (71-73). A similar ERR α expression plasmid has been previously shown to be incapable of activating transcription (8, 9). ERR α 1₁₋₃₇₆ failed to activate transcription in the presence of *c-erbB2*_{Onc} (Fig. 7, 10 vs. 4 and 8). Moreover, the presence of ERR α 1₁₋₃₇₆ inhibited full-length ERR α 's (ERR α 1₁₋₄₂₃) activation potential with *c-erbB2*_{Onc} (Fig. 7, 11 and 12 vs. 4). Therefore, I tentatively conclude that (i) ERR α 1₁₋₃₇₆ can act as a dominant-negative mutant, and (ii) *c-erbB2* signaling through the MAPK pathway targets ERR α to modulate its activation potential.

Upon original consideration of my fellowship application, the Review Panel suggested that rather than generating ERR α knockout cells, I should consider an antisense approach because of the likelihood of multiple endogenous ERR α alleles. However, the mechanisms whereby an antisense RNA interferes with translation are largely undefined and often do not satisfactorily block protein expression of a given target. I interpret the Panel's suggestion to mean that I should consider generating the equivalent of ERR α knockout cells using an approach whereby the caveat of potential multiple ERR α alleles are circumvented. Towards this end, I will employ the dominant-negative ERR α 1₁₋₃₇₆ variant. In light of the recent data described directly above and the Panel's Comments, rather than generating ERR α gene knockout sublines using matched parental WS8 and 5C cells as originally proposed, I would like to revise the original **Specific Aim II** as follows:

Revised Specific Aim II - To test whether *c-erbB2* induced activation of ERR α -dependent transcription may contribute to the TAM-resistant phenotype by generating and characterizing MCF-7 sublines stably-transfected with plasmids inducibly expressing wild-type or dominant-negative ERR α variants in the presence and absence of activated *c-erbB2*.

Revised Task II. Establishment MCF-7 cell sublines that inducibly express ERR α variants in the presence and absence of activated *c-erbB2*, thereby facilitating examination of ERR α 's potential role in a defined TAM-resistant cell line model system (months 15-36).

Revised Task 2A. Establishment of MCF-7 cell sublines inducibly expressing ERR α variants in the presence and absence of activated *c-erbB2* (months 15-24).

Matched stably transfected MCF-7 cell lines will be established that inducibly express wild-type or dominant-negative ERR α 1 variants with or without activated *c-erbB2* under the control of a Tetracycline (Tet) -regulated bi-directional promoter and are referred to herein as *erbB2*⁺/ERR α 1_{WT}, *erbB2*⁺/ERR α 1_{DN}, *erbB2*⁻/ERR α 1_{WT}, and *erbB2*⁻/ERR α 1_{DN} cells, respectively. These stably-transfected cell lines will express the transgenes in an inducible manner using a variant of the Tet repressor that

binds DNA when occupied by Tet to repress transcription (Tet-off repressor), but does not bind DNA when unoccupied by Tet (74). An MCF-7 cell line that stably expresses the Tet-off repressor (MCF-7/Tet-off) is available from Clontech (Palo Alto, CA). This MCF-7/Tet-off cell line has been shown to induce a Tet-regulated luciferase reporter gene 75-fold when Tet is removed from the culture medium. I will employ the bi-directional Tet-regulated expression plasmid pBI [Clontech;(75)] that allows two genes of interest to be regulated coordinately in a Tet-responsive manner through seven Tet-repressor DNA-binding sites flanked on both sides by minimal cytomegalovirus (CMV) promoters. Activated *c-erbB2* in combination with $ERR\alpha_{1WT}$ or $ERR\alpha_{1-376}$ (dominant-negative variant, $ERR\alpha_{1DN}$) will be subcloned into pBI to generate pBI-*erbB2*/ $ERR\alpha_{WT}$, pBI-*erbB2*/ $ERR\alpha_{DN}$, pBI- $ERR\alpha_{WT}$, and pBI- $ERR\alpha_{DN}$. I will stably transfect MCF-7/Tet-off cells with these bi-directional Tet-regulated expression plasmids to generate sublines in which $ERR\alpha$ is predicted to activate (*erbB2*⁺ sublines) transcription (Fig. 7) or repress (*erbB2*⁻ sublines) transcription (Fig. 6B). The pBI-based vectors will be co-transfected with a hygromycin-resistance expression plasmid to facilitate selection of the stably transfected sublines. I will screen the *erbB2*⁺ sublines for significantly greater MAPK activity compared to the *erbB2*⁻ sublines by immunoprecipitating the MAPK enzyme from these sublines and incubating it with the positive control substrate, PHAS-I, in the presence of [γ -³²P]ATP. Subsequently, these cell lines will be further screened for expression of equivalent amounts of exogenous $ERR\alpha$ variants by Western blotting methods employing the ligand affinity-purified anti- $ERR\alpha_{149-66}$ antibodies.

Using similar methods as those described for Fig. 6 and Fig 7, I will assay expression of pEREx3-luc and pSFREx3-luc reporter genes in *erbB2*⁻/ $ERR\alpha_{1WT}$ and *erbB2*⁺/ $ERR\alpha_{1WT}$ cells before and after withdrawal of Tet from the media to induce transgene expression. I predict that induction of $ERR\alpha_1$ expression should result in repression of ERE- and SFRE-regulated transcription in the *erbB2*⁻/ $ERR\alpha_{1WT}$ subline, whereas induction of both *c-erbB2* and $ERR\alpha_1$ expression should give rise to increased reporter gene activity in the *erbB2*⁺/ $ERR\alpha_{1WT}$ subline. The $ERR\alpha$ dependence of the effects on transcription will be verified by performing similar reporter gene assays in the control *erbB2*⁻/ $ERR\alpha_{1DN}$ and *erbB2*⁺/ $ERR\alpha_{1DN}$ sublines. As an additional control for *c-erbB2*-induced modulation of $ERR\alpha$ -dependent transcription through the MAPK pathway, similar co-transfection reporter gene assays will also be performed with the inclusion of a dominant-negative *Hras* construct (pJR*Hras*_{DN}). These inducible MCF-7 sublines will be used in **Revised Task 3B** to examine the modulatory effects of $ERR\alpha$ on *E2*-responsive transcription and to examine *c-erbB2* induced activation of $ERR\alpha$ on transcriptional regulation of genes whose promoters contain putative $ERR\alpha$ -binding sites (see Table 2 and Fig. 9).

Revised Task 2B. Examination of $ERR\alpha$'s potential role in *c-erbB2*-mediated TAM-resistant MCF-7 sublines (months 25-36).

Since MCF-7 cells overexpressing *c-erbB2* have been previously reported to acquire TAM resistance (61, 62), I will test by cell culture growth curve analysis multiple *erbB2*⁺/ $ERR\alpha_{1WT}$ sublines, cultured in the absence of Tet to induce *c-erbB2* expression, for *c-erbB2*-dependent attenuation of 4OHT's growth suppressive effects. However, long-term induction of *c-erbB2* expression may be required to attenuate sufficiently 4OHT's effects. If I observe this attenuation of 4OHT-mediated growth suppression or TAM resistance, I will likewise evaluate *erbB2*⁺/ $ERR\alpha_{1DN}$ cells to test whether $ERR\alpha_{1DN}$ blocks *c-erbB2*'s ability to promote TAM resistance. If *erbB2*⁺/ $ERR\alpha_{1DN}$ cells are not able to acquire TAM-resistance, this result would suggest $ERR\alpha$, probably in an activated form, may be involved in the development of some TAM-resistant breast tumors. If $ERR\alpha_{1DN}$ is implicated in interfering with *c-erbB2*-dependent acquisition of TAM resistance, this finding would suggest that activated $ERR\alpha$ may functionally bypass 4OHT's antagonism of $ER\alpha$ -dependent growth stimulation. In future studies, I would extend these findings by testing whether *erbB2*⁺/ $ERR\alpha_{1WT}$ cells grown as solid tumors in athymic mice frequently develop TAM resistance, whereas *erbB2*⁺/ $ERR\alpha_{1DN}$ cells do not.

Revised Task 2C. Characterization of $ERR\alpha$'s phosphorylation status (months 25-36).

Because *c-erbB2* signals through the MAPK pathway, I reasoned that activation of $ERR\alpha$ -dependent transcription in the presence of activated *c-erbB2* (Fig. 7) may be mediated by changes in $ERR\alpha$'s phosphorylation state. To begin to address this issue, I tested whether activated MAPK can directly phosphorylate $ERR\alpha$ *in vitro*. As a source of activated MAPK, I used rat p42 ERK2 (Calbiochem) that had been previously phosphorylated *in vitro* by a constitutively active MEK1 (MAPK

kinase) mutant. GST-fusion proteins were expressed and purified from *E. coli*. Activated ERK2 and [γ - 32 P]ATP were incubated with equimolar amounts of purified full-length ERR α 1 (GST-ERR α 1₁₋₄₂₃), truncated ERR α 1 variants (GST-ERR α 1₁₋₃₇₆, GST-ERR α 1₁₋₁₇₃) and control substrates. PHAS-1 [phosphorylated heat- and acid-stable protein (Calbiochem)] and GST- β globin served as positive and negative controls, respectively. Following the kinase reactions, the mixtures were resolved by denaturing PAGE and scanned with a PhosphorImager (Molecular Dynamics; Fig. 8). As expected, ERK2 phosphorylated PHAS-1, but not GST- β globin. I found all of the ERR α 1 variants were phosphorylated. Although this kinase assay is not quantitative, the increased signal intensity of GST-ERR α 1₁₋₄₂₃ versus GST-ERR α 1₁₋₃₇₆ and GST-ERR α 1₁₋₁₇₃ suggests ERR α 1 may be phosphorylated within the region 377-423 in addition to 1-173. Thus, ERR α can serve as a MAPK substrate *in vitro*.

Since *c-erbB2* can induce activation of ERR α -dependent transcription (Fig. 7) and MAPK can phosphorylate ERR α (Fig. 8), I propose that changes in ERR α 's phosphorylation state may regulate its transcriptional activity. Since MAPK-dependent phosphorylation of ER α results in loss of corepressor binding (76) and SMRT can interact with ERR α (O'Reilly, Ariazi, and Mertz, in preparation), changes in ERR α 's phosphorylation state may regulate its transactivation potential through differential recruitment of coregulators. I will determine ERR α 's phosphorylation status in MCF-7 cell lines inducibly expressing *erbB2*⁺/ERR α 1 and *erbB2*⁻/ERR α 1 and map the phosphorylated residues. In future studies beyond the scope of this Fellowship Proposal, my lab group will likely determine whether changes in ERR α 's phosphorylation state cause differential recruitment of coactivators and corepressors. To determine whether ERR α is differentially phosphorylated in *erbB2*⁺/ERR α _{WT} cells compared to *erbB2*⁻/ERR α _{WT} cells and to map the targeted residues, I will initially immunoprecipitate ERR α proteins from these sublines using the ligand affinity-purified anti-ERR α 1₄₉₋₆₆ antibodies, followed by separation of ERR α isoforms in 2D polyacrylamide gels in which the gel's pI gradient dimension should allow resolution of differentially phosphorylated ERR α isoforms. The 2D gels will be transferred to membranes for Western blotting analysis with anti-ERR α 1₄₉₋₆₆ antiserum to confirm the identity of ERR α . As additional controls, ERR α isoforms corresponding to various phosphorylation states in the 2D gels will be confirmed by co-migration of ERR α proteins treated with protein phosphatases and activated MAPK using methods similar to those used for Fig. 8. Later, ERR α phosphopeptides will be identified by radiolabeling cells with [γ - 32 P]ATP, immunoprecipitating ERR α using the anti-ERR α 1₄₉₋₆₆ antibodies, digesting the protein with trypsin, and resolving the peptides in a 2D polyacrylamide gel. Radiolabeled peptide spots will be recovered from the gel and sequenced using a triple quadrupole mass spectrophotometer available at the UW-Biotech Center. Thus, I will be able to identify the specific phosphorylation events that may regulate ERR α 's transcriptional potential as an activator or repressor due to the presence or absence of activated *c-erbB2*.

Upon review of this Fellowship Proposal, Reviewer B commented that generation of phosphospecific anti-ERR α antibodies might be a difficult and time-consuming undertaking. Hence, generation of such phosphospecific antibodies will likely not be able to be accomplished during the time of this award. However, my lab group intends to accomplish this task following the current granting period. In future studies beyond this Fellowship Proposal, following identification of phosphorylated residues, my lab intends to introduce amino acid substitutions to test whether these specific phosphorylation events are important for determining ERR α 's transcriptional potential by transient-transfection and reporter gene assays. If these phosphorylation events are important in determining ERR α 's transactivation potential, we would be encouraged to investigate in future studies whether ERR α 's phosphorylation status modulates the recruitment of corepressors and coactivators. If phosphorylation events modulate ERR α 's activities, we would eventually develop a phosphospecific anti-ERR α antibody that could distinguish activated from unactivated ERR α . Such phosphospecific antibodies might enable diagnostic determination of ERR α 's phosphorylation state in clinical tumors and examination for correlations between ERR α 's phosphorylation state and prognosis, sensitivity to antiestrogen therapy, and progression to TAM resistance. This concept has already been employed successfully with a phosphospecific anti-*c-erbB2* antibody (PN2A) where it was demonstrated by multivariate analyses that phosphorylated *c-erbB2* provided significant prognostic value in node-positive primary breast carcinomas (77).

Specific Aim III - To test whether genetic ablation of ERR α modulates the activity of transcriptional regulatory elements in the hormonally sensitive and insensitive cell lines described in Aim II. (A) ERR α -mediated transcriptional regulation will be investigated with heterologous reporter genes containing various EREs from promoters known to affect breast cancer, e.g. progesterone receptor (PgR), pS2, HER-2/neu, et cetera, as well as documented AP-1 sites modulated by ER α . (B) These EREs and AP-1 sequences will also be examined by gel-mobility-shift assays (GMSAs) and immunoshift assays. DNA binding and protein-protein interactions of ER α and hERR α 1, including possible specific isoforms, will be correlated with transcriptional activity.

Task 3. To test whether genetic ablation of ERR α modulates ERE- or AP-1-dependent transcription, and to correlate transcription activity with DNA binding and protein-protein interactions of ER α and ERR α , including possible specific isoform(s) (months 25-36)

Task 3A. Characterization of ERE and AP-1 DNA-binding activity by GMSAs and immunoshift assays (months 25-36).

Task 3B. Evaluation of ERE- and AP-1-dependent transcription using heterologous reporter genes (months 25-36).

ERR α and ER α bind EREs and SFREs [Figs. 3, 9 and 11; (4, 9)], directly interact through protein-protein contacts [Fig. 10; O'Reilly, Ariazi, and Mertz, in preparation; (4)], and are themselves estrogen-regulated genes [Fig. 1; (23)]. Thus, ERR α could modulate estrogen responsiveness through several non-mutually exclusive mechanisms. ERR α and ER α may compete for binding to the same element through protein-DNA interactions (model I; Fig. 11), bind separate subelements within a composite element [model II; (10)], form true ERR α -ER α heterodimers capable of binding DNA (model III), form ERR α -ER α complexes not capable of binding DNA (model IV), and/or compete for binding co-regulators of ER α (model V). Moreover, estrogen-dependent changes in ERR α and ER α expression (e.g., Fig. 1) may indirectly affect response element occupancy. ERR α has been demonstrated to modulate estrogen-responsive transcription of lactoferrin (10) and osteopontin (9, 78, 79). Thus, it is highly likely ERR α modulates other estrogen-responsive genes, including ones involved in breast cancer. Identification of ERR α -regulated genes and elucidation of the mechanisms by which it regulates these genes could impact the management of breast cancer and lead to the development of ERR α as a new therapeutic target.

Upon the review of this Fellowship Proposal, both Reviewers A and B commented that there were too many proposed experiments. Hence, I would like to alter **Specific Aim III** to focus on the characterization of DNA sequences directly bound by ERR α such as EREs and SFREs rather than indirectly bound by the receptor through protein-protein interaction such as AP-1 sites. Hence, I would like to revise the originally proposed **Specific Aim III** as follows:

Revised Specific Aim III -To Begin to Elucidate Mechanisms By Which ERR α May Play Roles in Breast Carcinogenesis By Identifying Estrogen-responsive Breast Cancer Prognosticator Genes Which Are Transcriptionally Modulated Through ERR α and Determining the Effects of ERR α on Transcriptional Regulation of These Genes.

Revised Task 3A. Characterization of putative ERR α -binding sites by GMSAs and immunoshift assays (months 25-36).

Identification of ERR α -binding sites in promoters of genes implicated in breast cancer.

Knowing which genes are transcriptionally modulated by ERR α may help to clarify ERR α 's potential role in breast cancer. To identify potential ERR α -responsive genes, the transcriptional regulatory regions of genes known to be implicated in breast cancer were examined for sequences potentially capable of being bound by ERR α by searching a eukaryotic promoter database [<http://www.epd.isb-sib.ch/>; (80)] and Genbank (<http://www.ncbi.nlm.nih.gov/>) (Table 2). The core ERR α -binding sequences used to query the databases were taken from studies conducted by the laboratories of J.M. Vanacker (8) and V. Giguère (7). ERR α binding to many of these potential sites was

confirmed with EMSAs employing lysates of COS cells transfected with an ERR α 1 expression plasmid (Fig. 9). Although not quantitative, these preliminary EMSAs clearly indicate that ERR α binds these sites to varying degrees. Almost all of the genes tested whose promoters contain confirmed ERR α -binding sites are estrogen responsive and exhibit prognostic significance in breast cancer (Table 2). I propose to characterize the DNA-binding properties of ERR α to many of these binding sites and to test whether some of the corresponding genes are responsive to changes in ERR α expression in **Revised Aim IIB**.

Cross talk between ER α and ERR α

One model by which ERR α can affect estrogen responsiveness is via the formation of heterodimers with ER α , thereby affecting ER α 's DNA binding specificity and/or affinity for EREs and SFREs. My lab group (4) and others (10) previously reported that ERR α and ER α directly interact. To map the region of ERR α that interacts with ER α , we used GST pull-down assays that employed GST-ERR α truncation variants and [³⁵S]-labeled ER α . We found that ER α interacts with a region overlapping ERR α 's T/A box located in the D domain (amino acids 145-173, Fig. 10; O'Reilly, Ariazi, and Mertz, in preparation). An amino acid sequence alignment of the T/A boxes from ER α , ERR α , ERR β , and ERR γ reveals 100 % conservation among the ERRs and 50 % conservation between ER α and the ERRs. Thus, ERR α binding to ER α is likely of physiological significance.

Models by which ERR α can affect estrogen responsiveness include (i) competing with ER α for binding to EREs and SFREs via direct protein-DNA interactions, and (ii) affecting ER α 's ability to bind DNA via direct protein-protein interactions. Intriguingly, such interactions could provide an additional level of modulation of receptor-dependent transcriptional activity by sequestering either receptor away from DNA. My lab group have found that the addition of increasing amounts of ERR α both interferes with ER α binding to the vitERE and results in the appearance of ERR α -DNA complexes (Fig. 11). However, either of these non-mutually exclusive models can account for this finding. We will distinguish between these two hypotheses using ERR α mutants that interact with ER α but do not bind DNA in EMSAs. The results of these studies will also likely provide a biochemical basis for the potential prognostic significance of the ERR α /ER α ratio correlating with DNA ploidy and S-phase fraction (Table 1).

Revised Task 3B. Evaluation of ERR α -binding sites using heterologous reporter genes (months 25-36).

ERR α -mediated repression of E₂-induced transcription.

ERR α has been demonstrated to activate (8-10, 34, 79, 81, 82) or repress (4) transcription depending upon the specific promoter and cell line. Given ERR α and ER α directly interact and bind many of the same HREs, ERR α could either interfere or enhance estrogen-responsive transcription. To test directly the affects of ERR α , I have developed reporter gene assays. My vectors contain 3 tandem consensus EREs or SFREs cloned upstream of a basal thymidine kinase (TK) promoter driving luciferase expression. They are termed pEREx3-luc (obtained from V.C. Jordan's laboratory) and pSFREx3-luc (obtained from J.M. Vanacker's laboratory). I co-transfected pEREx3-luc with an ERR α 1 expression vector into CV-1 cells, which naturally lack ER α , and MCF-7:WS8 cells, which contain ER α . As expected, ERR α 1 was found to activate transcription through the EREs in an E₂-independent manner in CV-1 cells (see Fig. 6A). However, ERR α 1 repressed both basal ERE-driven transcription and, importantly, E₂-induced transcription in MCF-7:WS8 cells (Fig. 6B).

Characterization of ERR α 's direct effects on transcription of the genes whose promoters contain validated ERR α -binding sites using reporter gene assays with inducible MCF-7 cell lines established in Revised Specific Aim IIA.

In **Revised Specific Aim IIA**, I propose to establish stably transfected MCF-7 cell lines that inducibly express specific ERR α 1 variants with or without c-erbB2_{Onc} under the control of a Tetracycline (Tet) -regulated bi-directional promoter where ERR α should activate (*erbB2*⁺/ERR α 1) transcription as in Fig. 7 or repress (*erbB2*/ERR α 1) transcription as in Fig. 6B. I will use the inducible MCF-7/*erbB2*⁺ cell lines to characterize the effects of ERR α -mediated modulation with various ER α -ligands of some of the genes whose promoters listed in Table 2 contain validated ERR α -binding sites. Next, I will examine in MCF-7/*erbB2*⁺ cells the effects of c-*erbB2*-induced activation of ERR α on transcriptional regulation of these estrogen-responsive genes. One of the many possible findings is that

the presence of activated *c-erbB2* will result in a reversal of $\text{ERR}\alpha$'s transcriptional potential on a given promoter: in cases where $\text{ERR}\alpha$ was a repressor it becomes an activator, but in cases where it was an activator it becomes a repressor. A second possibility is that the presence of activated *c-erbB2* will result in constitutive activation of $\text{ERR}\alpha$, independent of promoter context. A third possibility is that *c-erbB2*-induced alterations in $\text{ERR}\alpha$ may not result in a change in $\text{ERR}\alpha$'s transcriptional potential for some of the promoters. This last possibility would suggest $\text{ERR}\alpha$'s transcriptional potential, in certain cases, can be largely determined by promoter context independent of *c-erbB2*-induced activation of the MAPK signaling pathway. Later, I will study the effects of $\text{ER}\alpha$ ligands on *c-erbB2*-induced activation of $\text{ERR}\alpha$ by reporter gene assays as outlined directly above. In *erbB2*⁻/ $\text{ERR}\alpha$ 1_{WT} cells, I expect $\text{ERR}\alpha$ to interfere with E_2 -stimulated transcription. However, in E_2 -treated *erbB2*⁺/ $\text{ERR}\alpha$ 1_{WT} cells, the presence of activated *c-erbB2* leading to activation of $\text{ERR}\alpha$ may allow $\text{ERR}\alpha$ and $\text{ER}\alpha$ to cooperate either additively or synergistically. Treatment of these cells with ICI-182780, a complete antiestrogen, may block $\text{ER}\alpha$'s ability to cooperate with activated $\text{ERR}\alpha$ induced by *c-erbB2*. The possible outcomes of treating *erbB2*⁺/ $\text{ERR}\alpha$ 1_{WT} cells with the partial antiestrogen 4OHT are difficult to predict because *c-erbB2*-mediated stimulation of MAPK signaling may result in loss of corepressor binding with 4OHT-occupied $\text{ER}\alpha$ (76); therefore, in this case, $\text{ERR}\alpha$ and $\text{ER}\alpha$ may or may not cooperate. Similarly, I will employ the matched $\text{ERR}\alpha$ 1_{DN} cell lines to control for $\text{ERR}\alpha$ -dependent effects on transcription. Additional controls will include promoters containing site-specific mutations in their $\text{ERR}\alpha$ -binding sites. The data obtained from these reporter gene assays should indicate whether the identified binding sites are authentic $\text{ERR}\alpha$ response elements. Moreover, these transcription assays will provide a means to evaluate the functional activities of any potential $\text{ERR}\alpha$ variants identified in **Revised Specific Aim IIIA** above. It will also be interesting to see whether the effects observed differ among the various promoters tested.

In summary, I expect the data obtained from these experiments to validate the hypothesis that $\text{ERR}\alpha$ can affect the estrogen responsiveness of some genes via interactions with $\text{ER}\alpha$ and/or transcriptional response elements. They will likely also indicate that $\text{ERR}\alpha$ can directly affect expression of some genes whose altered expression correlates with breast cancer pathogenesis and disease progression.

KEY RESEARCH ACCOMPLISHMENTS

- ERR α is itself a modestly estrogen-responsive gene (Fig. 1).
- Ligand affinity-purified anti-ERR α ₁₄₉₋₆₆ antibodies have been generated and found suitable for Western blotting applications (Fig. 2) and EMSAs (Fig. 3).
- In a small sample size, ERR α mRNA is frequently expressed at significantly lower levels in random clinical primary breast carcinomas than in normal mammary tissues (Fig 4).
- Among a small number of clinical breast carcinomas, the relative ERR α /ER α mRNA ratio correlates with S-phase fraction and DNA ploidy. Thus, the ERR α /ER α ratio may serve as a biomarker of tumor aggressiveness (Table 1).
- At least one ERR α mRNA splice-variant exists in breast carcinomas (Fig. 5).
- ERR α can activate transcription in CV-1 and repress E2-dependent transcription in MCF-7 cells (Fig. 6).
- Increased c-*erbB2* signaling through the MAPK pathway can induce activation of ERR α (Fig. 7).
- ERR α can be phosphorylated by activated MAPK *in vitro* (Fig. 8).
- Potential ERR α DNA-binding sites have been identified in numerous promoters of genes implicated in breast cancer (Table 2) and have initially been authenticated by EMSAs (Fig. 9).
- ERR α directly interacts with ER α through protein-protein contacts mapped to ERR α 's T/A box adjacent to the receptor's core DNA-binding domain (Fig. 10).
- ERR α and ER α can compete for binding DNA (Fig. 11).

REPORTABLE OUTCOMES

Publications

O'Reilly, G. H., Ariazi, E. A., and Mertz, J. E. Human Estrogen-related Receptor α 1: Identification and Mapping of Functional Domains (manuscript in preparation).

Patents

Mertz, J. E., Johnston, S. D., Kraus, R. J., and Ariazi, E. A. Human Estrogen-related Receptor α . U.S. Patent (pending). Application Number 09/488,730. Filing date of January 20, 2000.

Awarded Funding

"Estrogen-related Receptor Beta in Breast Cancer and Estrogen Responsiveness"

Principal Investigator: Janet E. Mertz, Ph.D.

Co-principal Investigator: Eric A. Ariazi, Ph.D.

Agency: US Army Medical Research and Materiel Command

Type: Concept Award (BC995035) Period: 9/1/00 to 8/31/01

The aims of this proposal are: (1) to test whether alterations in ERRbeta expression correlate with the development of breast cancer; and (2) to begin to identify ERRbeta-regulated genes and to ascertain whether ERRbeta levels modulate estrogen responsiveness in mammary carcinoma cells.

Applied for Funding

"Estrogen-related Receptor alpha In Breast Cancer"

Principal Investigator: Janet E. Mertz, Ph.D.

Co-principal Investigator: Eric A. Ariazi, Ph.D.

Agency: National Cancer Institute, NIH

Type: R01 Period: 2001 to 2006

The aims of this proposal are: (i) to determine the utility of ERRalpha as a novel breast cancer prognosticator and target for therapy; and (ii) to elucidate the mechanisms by which ERRalpha modulates the transcriptional regulation of genes involved in breast cancer.

CONCLUSIONS

Several assays and reagents have been developed to study ERR α in breast cancer including real-time quantitative PCR assays, ligand-affinity purified anti-ERR α antibodies, EMSAs to investigate ERR α -binding to DNA, ERR α transient-transfection and reporter gene assay, and GST pull-down assays. I have found that ERR α is itself modestly estrogen responsive. I also found that expression of ERR α mRNA is significantly lower in a small panel of random breast carcinomas than normal MECs and that the ERR α /ER α ratio correlates with S-phase fraction and DNA ploidy, indicating potential as a novel breast prognosticator. Additionally, I found an ERR α 2 splice-variant mRNA in the breast tumors. To investigate ERR α -dependent transcriptional mechanisms, I have identified numerous potential ERR α -binding sites in the promoters of genes implicated in breast cancer and initially verified these sites by EMSAs. ERR α and ER α were demonstrated to compete for binding to a consensus ERE. However, the mechanism of this cross talk may occur through protein-DNA or protein-protein interactions since the receptors were also demonstrated to interact through a region containing ERR α 's T/A box (amino acids 145-173). ERR α activates transcription in CV-1 and represses E₂-stimulated transcription in MCF-7 cells. Furthermore, increased *c-erbB2* signaling through the MAPK pathway leads to activation of ERR α and ERR α can be phosphorylated by activated MAPK *in vitro*.

I had originally proposed establishing ERR α knockout sublines derived from matched hormonally-responsive and unresponsive MCF-7 cells. The Review Panel commented that I should try another approach such as antisense RNA methods to overcome the caveat of multiple endogenous ERR α alleles. I have since characterized a new ERR α truncation mutant that lacks a C-terminal coactivator binding domain that functions as a dominant-negative variant in transient-transfection and reporter gene assays. It has also been reported that stable-transfection of *c-erbB2* into MCF-7 cells results in a TAM-resistant phenotype, and I found that *c-erbB2* induces activation of ERR α . To study ERR α 's potential role in TAM-resistance, I therefore propose to establish MCF-7 sublines inducibly expressing wild-type and dominant-negative ERR α variants in the absence and presence of activated *c-erbB2*.

The above findings could provide the basis for future studies to determine whether ERR α status may indicate sensitivity to hormonal therapies, progression to tamoxifen resistance, and disease outcome.

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APPENDICES

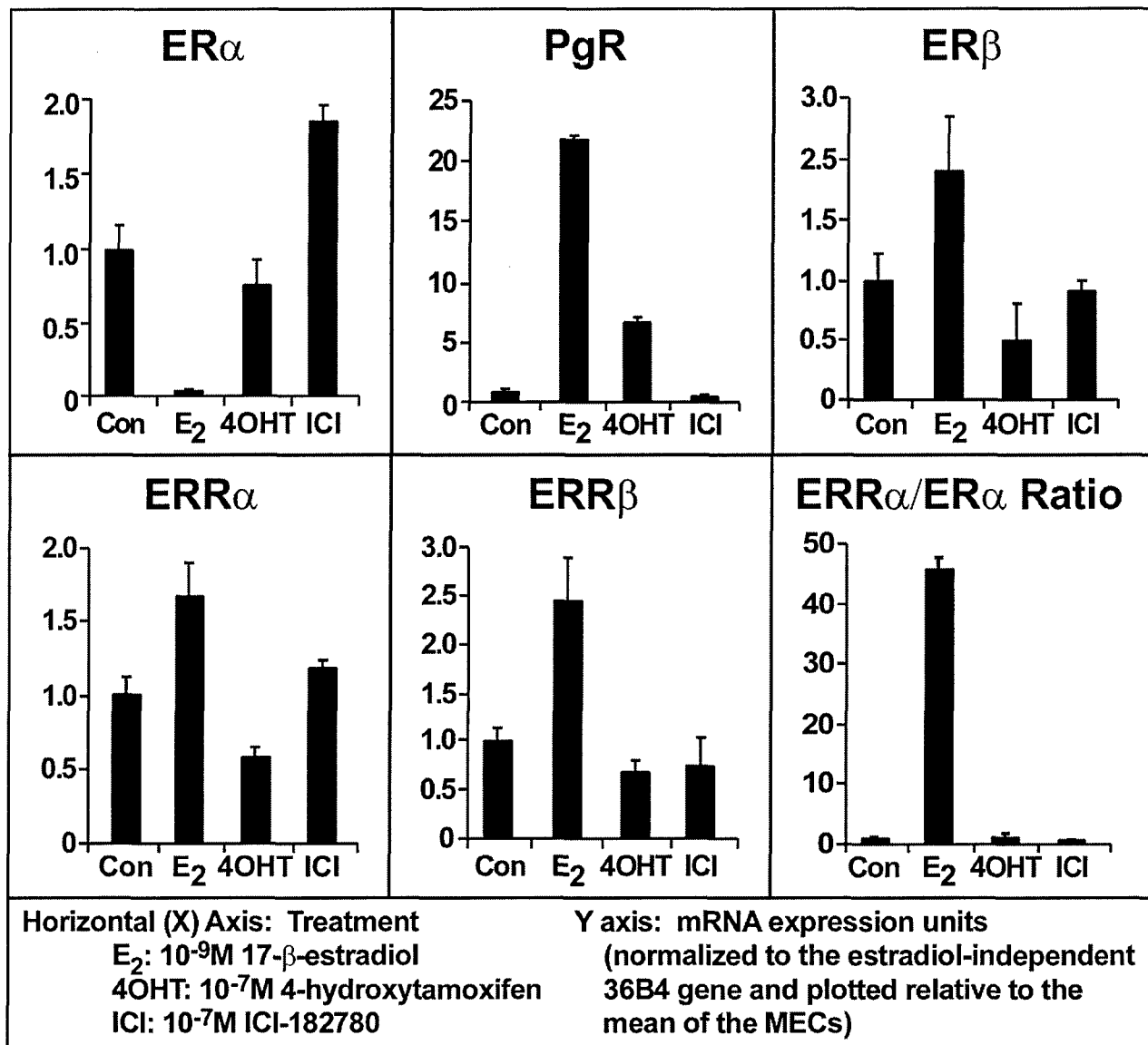


Fig. 1. Nuclear receptor levels in MCF-7:WS8 cells as determined by real-time quantitative PCR. Cells were seeded in estrogen-free medium [phenol red-free/dextran-coated charcoal-stripped fetal bovine serum (CS-FBS)] and treated with 10^{-8} M E_2 , 10^{-6} M 4OHT, 10^{-6} M ICI-182780, or vehicle alone (control) for 48 h. Error bars represent the SDs calculated from triplicate measurements on the same day. This experiment has been independently repeated with similar results.

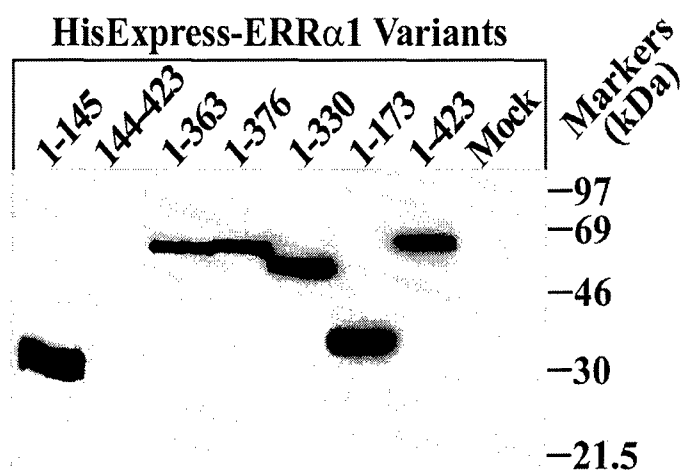


Fig. 2. Anti-ERR α ₁₄₉₋₆₆ antibody-probed Western blot of COS cells transfected with pExpERR α 1 [N-terminal “Express” (Invitrogen) epitope-tagged ERR α 1] corresponding to full-length (1-423) and truncated variants as indicated. The ERR α ₁₄₄₋₄₂₃ truncated variant does not contain the immunizing peptide sequence (ERR α ₁₄₉₋₆₆).

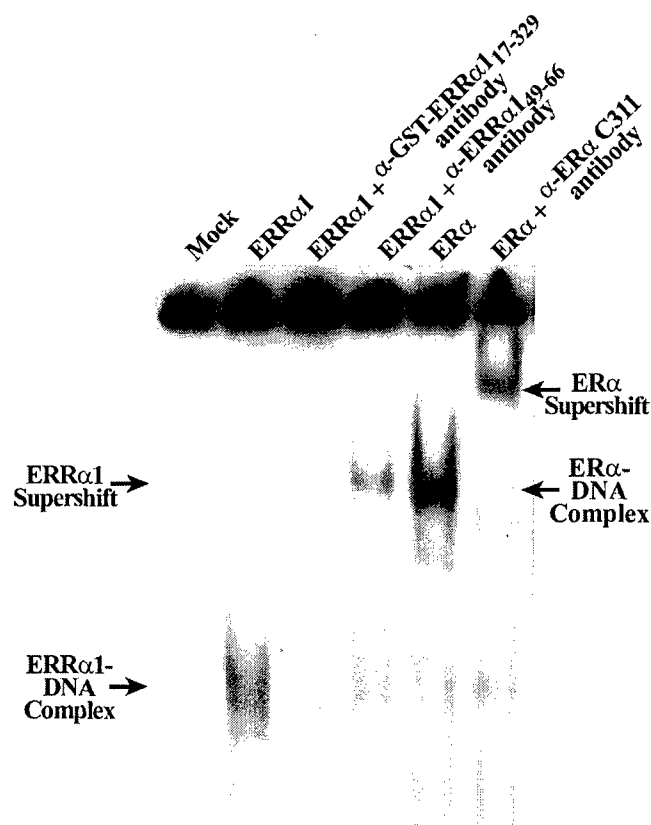


Fig. 3. EMSAs of ERR α and ER α binding to the vitERE probe DNA. Whole-cell extracts of COS cells transfected with pRSV-ERR α 1 or mock-transfected were used as protein sources.

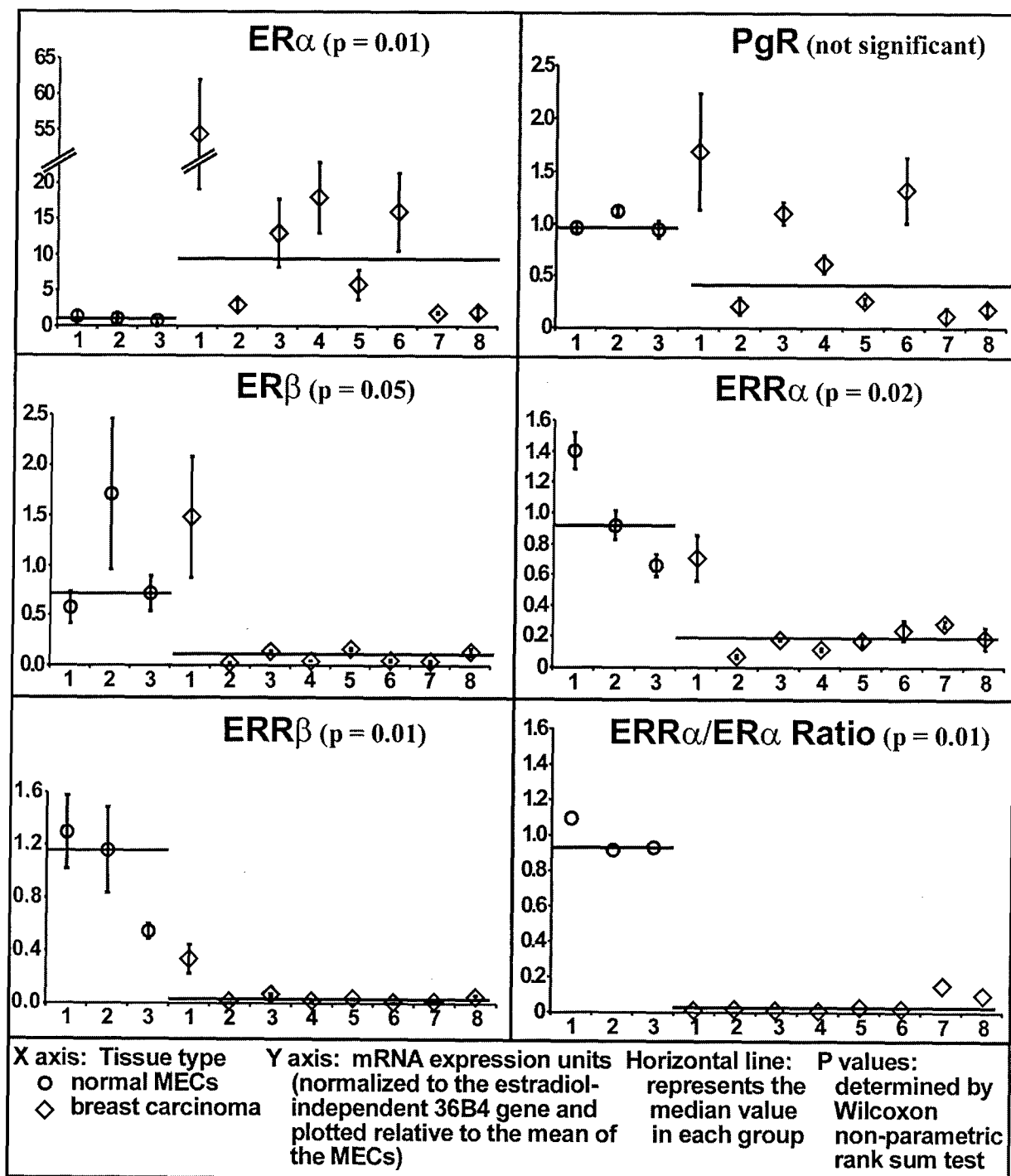


Fig. 4. Nuclear receptor mRNA levels in normal MECs and breast carcinomas as determined by real-time quantitative PCR. Error bars represent the SD of the mean from triplicate measurements on the same day.

	ER-LB	S-Phase	DNA Ploidy	ER α	ER β	ERR α	ERR β	ERR α /ER α
ER-LB	1.00 \ddagger	-0.50	0.39	0.83\ddagger	0.48	0.17	0.52	-0.76\ddagger
S-Phase	-0.50	1.00 \ddagger	-0.58	-0.50	-0.07	0.57	-0.14	0.71*
DNA ploidy	0.39	-0.58	1.00 \ddagger	-0.62	-0.17	-0.17	-0.17	-0.85\ddagger
ER α	0.83\ddagger	-0.50	0.62	1.00 \ddagger	0.52	0.19	0.33	-0.88\ddagger
ER β	0.48	-0.07	-0.17	0.52	1.00 \ddagger	0.43	0.81\ddagger	-0.17
ERR α	0.17	0.57	-0.17	0.19	0.43	1.00 \ddagger	0.52	0.14
ERR β	0.52	-0.14	-0.17	0.33	0.81\ddagger	0.52	1.00 \ddagger	-0.12
ERR α /ER α	-0.76\ddagger	0.71*	-0.85\ddagger	-0.88\ddagger	-0.17	0.14	-0.12	1.00 \ddagger

Table 1. Spearman's Rank Correlation Coefficients (ρ_s) for breast carcinoma properties. Statistical significance at ($P \leq 0.10$) *, ($P \leq 0.05$) \ddagger , and ($P \leq 0.01$) \ddagger . Significant correlation coefficients involving receptor mRNAs, other than with themselves, are bolded.

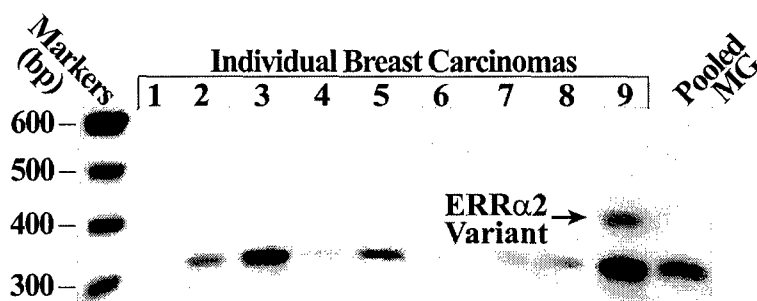


Fig. 5. ERR α 2 PCR products after 1 round of amplification in nine random primary breast carcinomas and a pool of eight normal mammary glands.

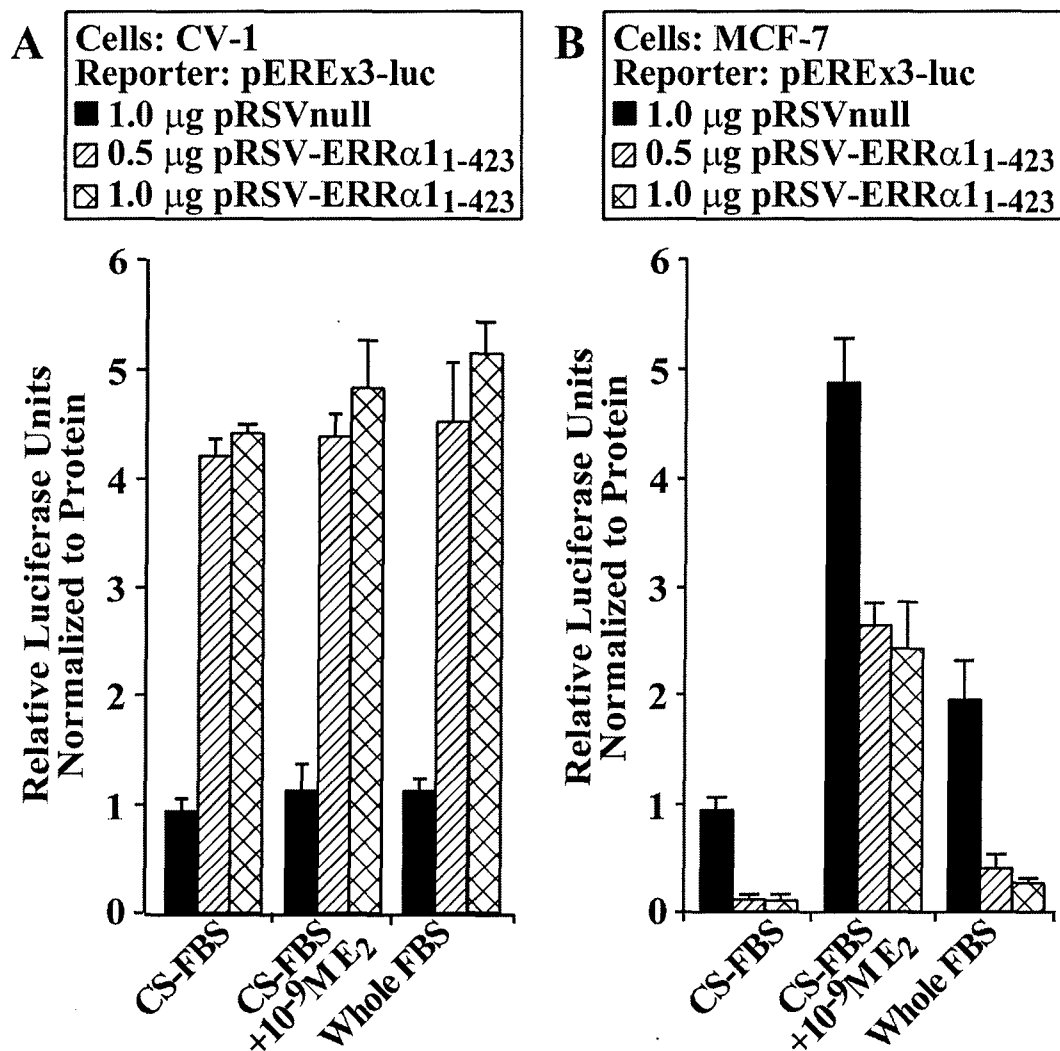


Fig. 6. ERR α activates transcription in CV-1 cells (**A**) and represses transcription in MCF-7 cells (**B**). Cells were seeded in 60-mm dishes in estrogen-free media two days prior to co-transfection with a vector encoding full-length ERR α 1 (pRSV-ERR α 1) and the reporter plasmid pEREx3-luc. Following co-transfection, cells were treated as indicated for 72 h and harvested for luciferase and protein assays. CS (charcoal-stripped), FBS (fetal bovine serum). Luciferase units were plotted relative to reporter gene activity in pRSVnull-transfected cells cultured in CS-FBS.

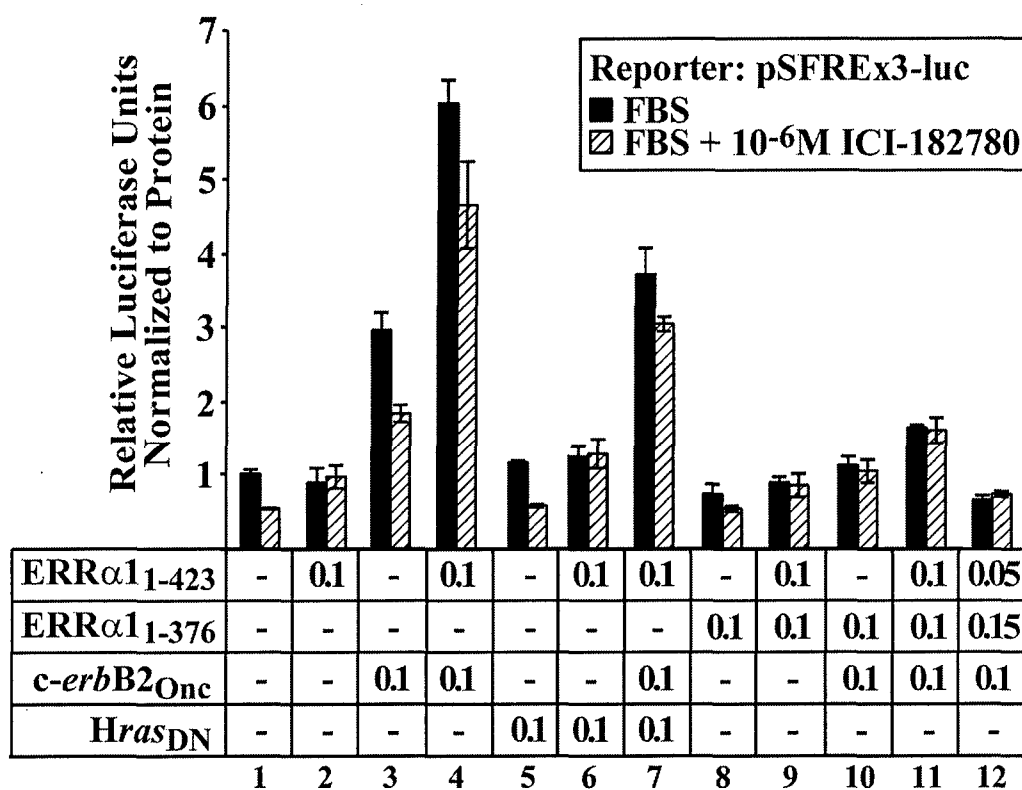


Fig. 7. Modulation of ERR α -transactivation potential through the MAPK pathway. MCF-7 cells were seeded in 22-mm dishes and co-transfected with pSFREx3-luc and the indicated amounts (in μ g) of expression vectors encoding N-terminally "Express"-epitope tagged (Invitrogen), full-length ERR α 1 (pExpERR α 1₁₋₄₂₃), C-terminal truncated ERR α 1 (pExpERR α 1₁₋₃₇₆), c-erbB2_{Onc} (pJRneu), and dominant-negative Hras (pJRHras_{DN}). Immediately following co-transfection, cells were treated with or without 10⁻⁶ M ICI-182780 and harvested after 48 h for luciferase and protein assays. Luciferase units were plotted relative to reporter gene activity in cells cultured without ICI-182780 (column 1).

PHAS-1
GST-ERR α 11-173
GST-ERR α 11-376
GST-ERR α 11-423
GST- β globin



Fig. 8. Autoradiogram of SDS-PAGE of products of MAPK phosphorylation reactions. Equimolar amounts of the indicated purified proteins were incubated with activated MAPK and [γ - 32 P]ATP.

Gene	Prognostic Status Reference(s)	E ₂ Response Reference(s)	Potential ERR α -binding Sequence	ERR α Bound [†]
Aromatase	-	-	cctgagactctaCCAAGGTCAgaaatgctgcaa	yes
BRCA1	(83-86)	(87-92)	gtaattgctgtaCGAAGGTCAgaatcgctacct	yes
BRCA2	(85, 86)	(92)	agaacatccctTTAAGGTCaacaaggat	yes
cathepsin D	(93, 94)	(95-101)	tggcatattgggTGAAGGTCaaggagtggtt	yes
cyclin D1	(102-106)	(107-109)	gcgaggaaagcgTGAAGGTGAttcagtaatt	yes
ER α	(1, 2, 110, 111)	(17, 18, 20, 112-)	atgtttggtatgAAAAGGTCacattttatattc	yes
ER β	(37, 47)	(24)	ggtgctccactTAGAGGTCacgcgcggcg	yes
EGF	(115-117)	-	caaataatgggcTGAAGGTGAactatcttact	ND
FGF-3/ <i>int-2</i>	(103, 118, 119)	-	acaggaaagcccCAAAGGACAgcagcaagagga	ND
<i>c-erbB2/HER-2/neu</i>	(110, 120-124)	(125-128)	aaaggaaacttcCCAAGGTCacagagctgagct	yes
IGF1	(129, 130)	(131)	aaaagaaaaaTCAAGGTCCaggttattcca	no
IGF2 site 1 (IGF2-1)	(132, 133)	(134, 135)	tgaggagcatttGAAAGGTGCctgtctgcaaac	no
IGF2 site 2 (IGF2-2)			ctgtcggcagggaACAAGGTCAccccttgccgtt	yes
IGF2 site 3 (IGF2-3)			ggtggacgctgcTGAAGGTGAgcgagaccccg	yes
IGF1R	(130, 136-139)	(140)	tcgcctcgctgTGACCTTCagcgagccggagc	yes
MDM2	(141-143)	(144)	gggagttcaggTAAAGGTCAcggggccggggc	yes
PgR site 1 (PgR-1)	(2, 110, 111, 145, 146)	(147, 148)	aaaattgtttgTCTAGGTCAtttgattttca	yes
PgR site 2 (PgR-2)			tccttgctaaacCCAAGGTCAtaaatcttttct	ND
PgR site 3 (PgR-31)			tcctaaggactgTCAAGGTCAtaaatcaagg	ND
Prl site 1 (Prl-1)	(149, 150)	(151)	tgccattttctTCTAGGTCAaccccaatgga	yes
Prl site 2 (Prl-2)			caaatttgaacTAAAGGTCAcaggctgcttta	yes
Prl site 3 (Prl-3)			cctcagagtggcTCAGGGTCagagaaggtagag	yes
Prl site 4 (Prl-4)			tgagagaaatgaTGAAGGTGAgatctgagacta	no
pS2 site 1 (pS2-1)	(152-154)	(155-161)	tccttccccctGCAAGGTCAcgggtggccacc	yes [‡]
pS2 site 2 (pS2-2)			gtaggacctggaTTAAGGTCAggttgaggaga	ND
RAR α	(91)	(162-164)	gaagtgacttgTCAAGGTCAcacagctctcag	ND
TERT	(165-174)	(175)	ccagctcctcaGGCAGGACcctgcgggggaa	yes

Table 2. Potential ERR α -binding sites located in the transcriptional regulatory regions of genes implicated in breast cancer. [†]ERR α -binding sites were confirmed using EMSAs as shown in Fig. 9. [‡]Data not shown. ND (not determined), BRCA (breast and ovarian cancer susceptibility gene), FGF (fibroblast growth factor), IGF (insulin-like growth factor), IGF1R (IGF1 receptor), MDM2 (human p53-binding protein murine double minute 2), Prl (prolactin), RAR α (retinoic acid receptor α), TERT (telomerase catalytic subunit gene).

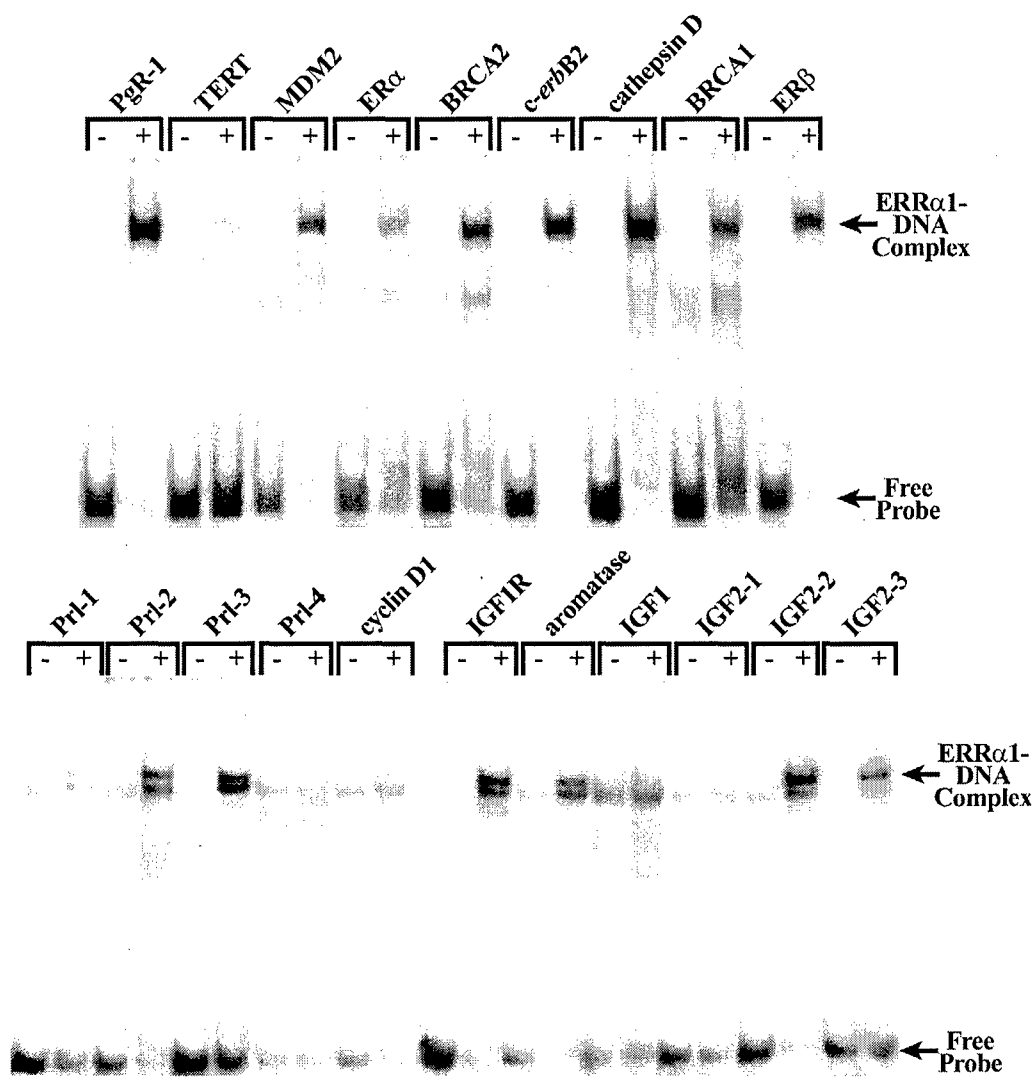


Fig. 9. EMSAs showing ERR α binding to SFREs located in the transcriptional regulatory regions of genes implicated in breast cancer listed in Table 2. Whole-cell extracts of COS cells transfected with pExpERR α 1 (+) or mock-transfected (-) were used as protein sources.

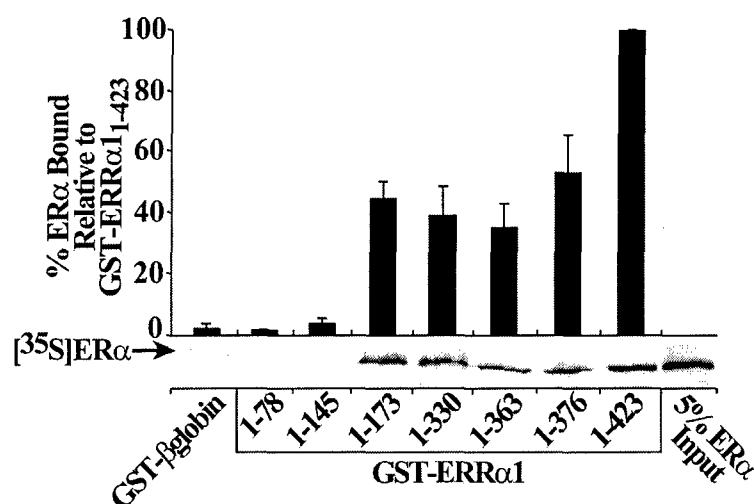


Fig. 10. GST pull-down assays of GST-ERR α 1 truncation variants interacting with [35 S]-ER α . GST-ERR α 1 variants were expressed and purified from *E. coli*. [35 S]-ER α was synthesized *in vitro* with rabbit reticulocyte lysates.

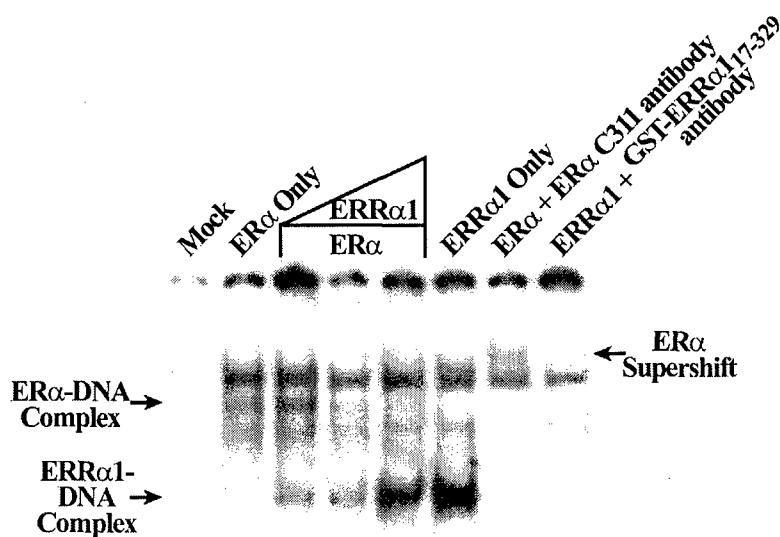


Fig. 11. Competition EMSAs between ERR α 1 and ER α for binding a vitERE probe DNA. Lysates of COS cells transfected with ERR α 1 and ER α expression plasmids were used as protein sources.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
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